First Archaeal rDNA sequences from coastal waters of Argentina: unexpected PCR characterization by using eukaryotic primers

Running title: First Archaea rDNA sequences from the Argentine Sea

Primeras secuencias de ADNr de Archaea en aguas costeras de Argentina: inesperada caracterización por PCR usando cebadores para eucariotas

Titulo corto: Primeras Secuencias de ADNr de Archaea del Mar Argentino

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ABSTRACT. Many members of Archaea, a group of prokaryotes recognized three decades ago, colonize extreme environments. However, new research is showing that Achaeans are also quite abundant in the plankton of the open sea, where are fundamental components that play a key role in the biogeochemical cycles. Although the widespread distribution of Archaea the marine environment is well documented there are no reports on the detection of Archaea in the Southwest Atlantic Ocean. During the search of picophytoplankton sequences using eukaryotic universal primers, we retrieved archaeal rDNA sequences from surface samples collected during Spring at the fixed EPEA Station (38º28'S-57º41'W, Argentine Sea). From environmental DNA and using PCR methodology, two DNA fragments of about 1,700 and 1,450 bp were visualized after electrophoresis in agarose gels, which were separately purified, cloned and sequenced. BLAST analysis showed that sequences of the highest size corresponded to eukaryotic organisms and, unexpectedly, those of about 1,460 bp corresponded to Archaeal organisms. Phylogenetic analysis showed that Archaeal sequences belong to Euryarchaeota of the Marine Group II, which is characterized as a methanogenic lineage. This is the first report on the presence of Euryarchaeota-Group II sequences in environmental water samples of the Argentine Sea. The fact that Archaea sequences were amplified with primers non specific for this group may suggest an unexpected abundance of these organisms in the early spring in the Argentine Sea.

Keywords: Euryarchaeota, Argentine Sea, environmental rDNA, PCR methodology, primer design

Resumen. Muchos miembros de las Archaea, un grupo de microroganismos descriptos hace aproximadamente treinta años, colonizan ambientes extremos. Sin embargo, las investigaciones más recientes han demostrado que las Archaeas también son abundantes componentes del plankton marino, siendo algunos grupos de Archaeas componentes fundamentales de los ecosistemas marinos debido a su rol clave en los ciclos biogeoquímicos. Aunque la ubiquidad de las Archaeas ha sido bien documentada, hasta el momento no hay reportes de la presencia de representantes de este grupo en el mar Argentino. En un estudio de biodiversidad orientado a determinar secuencias de picoeucariotas utilizando cebadores universales para eucariotas, encontramos secuencias de ADNr de Archaeas en muestras recolectadas durante la primavera en la estación fija EPEA (38º28'S-57º41'W, Mar Argentino). A partir de ADN ambiental y mediante el uso de la metodología de PCR, obtuvimos dos fragmentos de aproximadamente 1.700 y 1.460 bases, los cuales fueron separados y visualizados después de electroforesis en geles de azarosa, y luego purificados, clonados y secuenciados. El análisis del BLAST mostró que las secuencias de tamaño superior correspondían a organismos eucariotas y las secuencias de menor tamaño pertenecían a Archaea. El análisis filogenético mostró que las secuencias de Archaea se agrupan con Euryarchaeota Marina Grupo II, caracterizado como un linaje metanógeno. Éste es el primer reporte de la presencia de secuencias de Euryarchaeota-Grupo II en aguas del mar Argentino. El hecho de que las secuencias de Archaea hayan sido amplificadas con cebadores no específicos para este grupo, sugeriría una inesperada abundancia de estos organismos durante los inicios de primavera en el Mar Argentino.

Palabras clave: Euryarchaeota, Mar Argentino, ADNr ambiental, metodología PCR, diseño de cebadores.

INTRODUCTION

Microbial diversity is clearly a topic of considerable importance and interest. In the past decades the most surprising discoveries in biodiversity arose from studies on the distribution of microbial communities in the ocean. Marine ecosystems are continually subject to oscillations in environmental conditions. It is now widely recognized that climate change and biodiversity are interconnected (Bowland 2006). Because global warming is expected to have a significant influence on hydrologic cycle over the next several centuries and thus on species composition, the analysis of the current biodiversity is urgent. Increasing amount of knowledge has been reported in the last decades; however, the introduction of molecular methodologies and metagenomic analyses opened new avenues in the understanding of marine microbial diversity. Using these tools, the ubiquitous presence of completely novel lineages, with no representatives in cultures, has been established for the three domains of life: Bacteria (Giovannoni *et al*. 1990), Archaea (Delong 1992, Fuhrman *et al*. 1992), and more recently Eukaryota (Díez *et al*. 2001, López-García *et al*. 2001a, Massana *et al*. 2002, Romari and Vaulot 2004, Groisillier *et al*. 2006, Lovejoy *et al*. 2006).

Achaeans are microscopic single-celled organisms that constitute a group of prokaryotes, recognized in 1977 as an independent monophyletic group. Although initially they were believed to be limited to anaerobic, hyperthermal, and highly saline habitats, they were also found in both marine and freshwaters environments (DeLong 1992, Fuhrman *et al*. 1992, Massana *et al.* 1997 and 1998, Murray *et al.* 1999, Massana *et al.* 2000, Karner *et al.* 2001, Auguet and Casamayor 2008). Thus, it is recognized that marine archaeal populations are diverse, complex and widespread (Danovaro 2010). There is now increasing evidence that marine Archaea make an important contribution to the biogeochemical nitrogen and carbon cycles (Bartossek *et al.* 2010).

Based on 16S rDNA phylogeny from cultivated organisms, marine Archaea are phylogenetically distributed through four main taxonomical clusters: one cluster of Crenarchaeota, the Marine group I (MGI), and three clusters of Euryarchaeota, group II, III and IV (Galand *et al*. 2009). Members of the marine Group I have a key role on the biogeochemical cycles, being a fundamental component of the marine ecosystem. Although Crenarchaeota consist mainly of thermophilic species, the genome *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus*, two non-thermophilic strains were completely sequenced (Preston *et al*. 1996, Könneke *et al*. 2005, Bartossek *et al*. 2010). Archaea of Group II of planktonic *Euryarchaeota* have more varied metabolisms (hence the name "eury-," meaning variable), but most biochemical studies have focused on methanogenesis, a unique property of some Archaea (comprising Halobacteriales, Thermoplasmales, Thermococcales, Sulfolobales, Pyrodictiales, Archaeoglobus, Methanobacteriales). Representatives of Group III are restricted to deep waters, having been found in waters below the photic zone (Galand *et al*. 2009). Group IV was first discovered by Rodriguez-Valera (1979) and sequences of its members were clearly distinct from all known planktonic Archaea (López García *et al*. 2001a).

Analyses of rDNA sequences from environmental samples have revealed that Archaea are ubiquitous and far more abundant than previously assumed (Stein and Simon 1996, Karner *et al*. 2001, DeLong 2003). Culture-independent techniques based on 16S rDNA analyses showed the existence of Archaea in the open-ocean, marine sediments, soils and freshwater lake sediments (Massana *et al*. 2000, Schleper *et al*. 2005, Galand *et al*. 2009, Bartossek *et al*. 2010). Particularly, marine Archaea have been shown to reside in coastal and offshore temperate and cold waters worldwide (DeLong 1992, Fuhrman *et al*. 1992, Massana *et al*. 1997, Galand *et al*. 2010). Karner et al. (2001) found that pelagic *Crenarchaeota* form North Pacific Ocean Gyre comprising more than 30% of the total

microbial cells from 200 m to 5,000 m. Herndl *et al.* (2005) estimated that at depth of 100 m *Euryarcheota* contributed about 17% of picoplankton abundance of the North Atlantic Sea while the contribution of *Crenarchaeota* was about 18.5%. Primers to detect archaeal sequences by PCR approach have been designed to amplify all prokaryotic 16S rDNA genes and are referred to as 'universal' (DasSarma and Fleischmann 1995, Reysenbach and Pace 1995, Vetriani *et al.* 1999, Baker *et al*. 2003, http://bioinfo.unice.fr/454/VC/archaea_primers_sorted_by_Fsequences.html) or for taxa specific detection (Baker *et al*. 2003). López-García *et al*. (2001 a and b) found Euryarchaeota sequences belonging to Marine Group II and III of the Antarctic Polar Front sea water by using and designed different primer sets for 16S rDNA amplification.

To our knowledge there are no reports on the detection of Archaea in the Southwest Atlantic Ocean. In this study, we report the presence of rDNA archaeal sequences in surface water samples of the Argentine Sea that constitute the beginning of more comprehensive studies to understand the contribution of prokaryotes to biogeochemical cycles of marine ecosystems.

MATERIAL AND METHODS

Sample collection

Water samples were collected by the cruises conducted monthly on-board the RV "Capitan Canepa" (INIDEP). Surface water samples were collected in September, October and November at the fixed EPEA Station in the Argentine Sea (38º28'S - 57º41'W at 27 nautical miles south of Mar del Plata, Argentina). Environmental characteristics of sampling site (photosynthetically active radiation, temperature and

salinity) were described by Silva *et al*. (2009). Water samples (2.5 liters) were taken using a bucket at the surface, prefiltered through a 25 µm pore size to eliminate microplankton components, passed through a polycarbonate membrane of 3 µm pore size (Nuclepore) to remove nanoplankton components, and finally filtered through a 0.2 µm pore size (Durapore). Filters were transferred into a cryovial tube, immediately frozen in liquid nitrogen, and stored at -80°C until nucleic acid extraction.

Nucleic acid extraction

Genomic DNA was extracted from marine samples (4 subsamples at each collect) according to standard protocols (Sambrook and Russell 2001). Nucleic acid extraction began with the addition of lysozyme (1 mg mL^{-1}) to the filter unit and incubation at 37°C for 30 min. Then, proteinase K (0.5 mg mL^{-1}) and sodium dodecyl sulfate (SDS, 1%) were added, and the filter was incubated for 2 h at 55°C. The lysate was recovered from the filter, which in turn was rinsed with 1 mL of lysis buffer (Tris-HCl 50 mM, pH 8; EDTA 40 mM, pH 8; sucrose 0.75 M; nuclease-free water) with lysozyme (1 mg/mL**)**. All centrifugations were performed at 13,000 rpm and at 4°C. After centrifugation for 7 min, the upper phase was transferred to a clean tube. The pooled lysates were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0) and once with chloroform-isoamyl alcohol (24:1). After removing any residual phenol by centrifugation, the aqueous phase was transferred into a new 1.5 mL tube containing 750 µL cold isopropanol and 1/10 volume sodium acetate (0.3 M final concentration, pH 5.2). Tubes were placed in -20°C freezer overnight. After a centrifugation for 30 min the supernatant was decanted into a beaker and the DNA pellet washed with 200 µL 70% ethanol at -20ºC. The DNA pellet was dried and re-suspended in 50 µL PCR water and

stored at -20°C until use. DNA concentration was determined by measuring absorbance at 260 nm.

PCR amplification, cloning and sequencing

Extracted DNA was used as template in PCR reactions using eukaryotic 18S ribosomal DNA (rDNA)-specific primers to Eukarya EukA 5´-AACCTGGTTGATCCTGCCAGT-3´; EukB 5´-TGATCCTTCTGCAGGTTCACCTAC-3´ (Medlin *et al*. 1988). The PCR conditions were as follows: initial DNA denaturizing for 3 min at 94°C, followed by 30 cycles of denaturizing for 45 s at 94°C, annealing for 1 min at 55°C, extension for 3 min at 72°C, plus one additional cycle with a final 10-min chain elongation at 72°C. The 25-µL reaction volume contained 50 ng of DNA and 5 pmol of each primer. Following amplification, the PCR products were analyzed by electrophoresis on a 0.8% agarose gel and DNA fragments were visualized with ethidium bromide. DNA from the agarose gel was extracted using the QIAGEN MinElute gel extraction kit. The purified PCR products were cloned into the pGemTeasy cloning Vector kit (Promega). The recombinant plasmid was inserted into *Escherichia coli* DH5α competent cells, which were grown in LB medium at 37°C for 20 min. Cultures were sprayed on LB/Ampicillin/IPTG/X-Gal (1 mL 100 mg mL^{-1} ampicillin, 0.12 g isopropyl-β-D-thiogalactopyranoside (IPTG) in 5 mL deionised water; 0.10 g 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal) in 2 mL N,N-dimethylformamide). Twenty colonies of each sample (a total of 100 positive white colonies) were separately grown (37°C, over night) in LB medium with ampicillin. The presence of rDNA inserts was confirmed by colony PCR using the same primers and amplification conditions. The PCR products were digested with the restriction endonuclease *Hae*III. All digestions were completed independently and performed in 15

μL of volume with 3 μL of PCR product, 10X buffer and 3 units of restriction endonuclease. The solution was incubated at 37°C for one hour. Digested samples were run by electrophoresis (80 V, 3 h) in agarose gels (2.5%) (Meta Phor. Cambrex Bio Science Rockland Inc. Me USA). Agarose gels were stained with ethidium bromide and the restriction fragment length polymorphism (RFLP) products were visualized with UV transillumination. Size of inserts was confirmed by *EcoR*1 restriction enzyme (Promega, Madison, USA) by plasmid digestion. The inserts of clones with different RFLP patterns were sequenced (Macrogen, Korea). Sequence was deposited in GenBank (BankIt1405479, uncultured HQ541865).

Phylogenetic analysis and rDNA thermodynamic properties prediction

Comparisons of rDNA sequences were performed using nucleotide sequences available in the databases at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were generated using the CLUSTAL W software and graphic representations of phylogenetic trees were performed using the MEGA4 software (Tamura *et al.* 2007). The trees were statistically evaluated with non-parametric bootstrap analysis (number of replicates = 1,000). The secondary structures for ribosomal RNA were predicted using RNADRAW (Vienna RNA package; http://www.rnadraw.com) and RNAfold program (available at http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

RESULTS AND DISCUSSION

In our study of picophytoplankton diversity of the Argentine Sea we used EukA/EukB primers to PCR amplify eukaryotic small subunit ribosomal rDNA genes (Medlin *et al*. 1988) from DNA extracted from surface-sea samples collected during spring in the Argentine Sea. Diez *et al*. (2001) had been demonstrated the specificity of EukA/EukB primer pair to construct clone libraries of eukaryotes and the ability of the primers to recover distant phylogenetically-related eukaryotic groups (Stramenopiles, Alveolates, Prasinophytes as well as Chrysomonad, Cercomonads and Fungi) from North Atlantic, Antarctica, and Mediterranean Sea surface waters. Moreover, those primers were successfully used in most recent biodiversity studies of marine picoeukaryotes (Groisillier *et al*. 2006, Guillou *et al*. 2008, Hoppenrath *et al*. 2009, Not *et al*. 2009). The analysis of the amplification products using EukA/EukB primers after separation by electrophoresis on agarose gel, revealed that while a DNA fragment of expected size (1,700 bp) was present in all the samples, an additional band of 1,460 bp was visualized in all samples collected in September and October (fig. 1a). About 66% of the analyzed samples showed the second band. The two DNA amplified fragments were separately purified and cloned in *E. coli* cells (fig. 1b). Further sequencing of the inserts revealed that nucleotide sequences of 1,700-bp bands corresponded to eukaryotic organisms. In the case of samples collected in September, the sequences were ascribed mainly to Stramenopiles (e.g. *Bolidomonas* sp.) and Alveolata (e.g. *Laboea* sp.) whereas those of October matched with sequences belonging to Stramenopiles (e.g. *Pedinella* sp.) and Chlorophyta Prasinophyceae (e.g. *Bathycoccus* sp.). Amplified DNA of samples collected in November produced only one band of about 1,700 bp and sequences matched with sequences belonging to Alveolata (e.g. *Noctiluca* sp.). Surprisingly, nucleotide sequences

of the 1,460-bp inserts corresponded to archaeal rDNA, whose RFLP patterns were compared (fig. 2). The sequences of the inserts matched with an uncultured marine Archaea grouped with the Marine Group II of Euryarchaeota (fig. 2).

The sequence alignments of Archaea 16S rDNA and 18S rDNA universal primers (EukA/EukB) used for PCR amplification confirmed that the primer pair shares 100 % identity with archaeal rDNA regions. Analyses with the SILVA rRNA database (http://www.arb-silva.de) indicated that EukA and EukB primers would amplify a very small number of Archaea sequences (2 out of 11,954 Archaea available sequences). Therefore, the fact that Archaeal sequences could be recovered from the environmental DNA with primers designed for eukaryotes suggests that some scarce Archaea strains of Marine Group II were very abundant at least during September and October, considering the obvious competition in the annealing step between Archaea and Eukaryotic sequences for the primers.

The identification of Archaea sequences led us to analyze the primers reported to specifically retrieve sequences of these organisms. We compare EukA-EukB sequences with the Archaea primers and with the more abundant sequence we obtained in this study (HQ541865) (table 1). Whereas some of the reported primers align with the HQ541865 sequence in more internal positions than EukA-EukB, others have poor or no complementation with HQ541865. Baker *et al*. (2003) proposed two new primer pairs (A571F 5'-GCYTAAAGSRICCGTAGC-3'/UA1204R 5'-TTMGGGGCATRCIKACCT-3' and A751F 5'-CCGACGGTGAGRGRYGAA-3'/UA1406R 5'-ACGGGCGGTGWGTRCAA-3') to amplify sequences from Crenarchaeote and Euryarchaeota type strains. Also Gantner et al. (2011) presented two new archaeal primers (340F 5'-CCCTAYGGGGYGCASCAG -3'and 1000R 5'-GGCCATGCACYWCYTCTC-3') which were designed from 8,500 aligned archaeal

sequences by using the SILVA database. They reported that designed primers showed a high archaeal specificity (< 1% bacteria amplification) covering 93 and 97% of available sequences for Crenarchaeota and Euryarchaeota respectively. However, these primers have a high level of degeneracy, which could lead to amplify non-target genes or domains. From non-degenerative primers used to the comparison (table 1), only one aligns with one of the primers used in this study. The specific eukaryotic EukA primer matches with EK4F primer, designed by Robb *et al*. (1995) and later reported as very high specific for methanogen sequences (Baker *et al*. 2003), but which does not match with any other Archaeal group. Remarkably, EukA primer has four additional bases than the EK4F which allowed the specific retrieval of the Marine Group II of Euryarchaeota sequences.

It has been shown that the two major groups of planktonic Archaea (Crenarchaeota and Euryarchaeota) might account for about one-third of all prokaryotic cells in the global ocean (Karner *et al*. 2001). Sequences of the Marine Group II of Euryarchaeota have been found in both, the Sta Barbara Channel, California, from 0 to 200 m (Massana *et al*. 1997) as well in the Artic and Antartic surface waters (Murray *et al.* 1999, Bano *et al.* 2004). Although they were reported as more abundant in surface waters in Pacific and Beaufort Sea, their presence was also reported in different oceanic regions sampled at depths between 5 and 200 m (Massana *et al*. 2000, Karner *et al*. 2001, Herndl *et al*. 2005, DeLong *et al*. 2006, Galand *et al*. 2009). In the South Atlantic Sea, however, the presence of Archaea remained undescribed until the present report.

Conclusion

Euryarchaeota sequences affiliating with the known Group II are recognized to be widespread in the oceans worldwide (Stein and Simon 1996, DeLong 2003) and this study contributes with the identification of the first 16S rDNA sequences belonging to Euryarchaeota-Marine Group II in environmental surface water samples of the Argentine Sea, where could play an important role in biogeochemical cycles. The casual way that led to these results, in turn, gives more weight to the finding, since it highlights the relative abundance of these Archaea in certain months of the year, and how unlikely it would have been to detect them using reported information.

Acknowledgments

This project was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, grant PICT 2002 N° 12233), INIDEP, CONICET, and FIBA.

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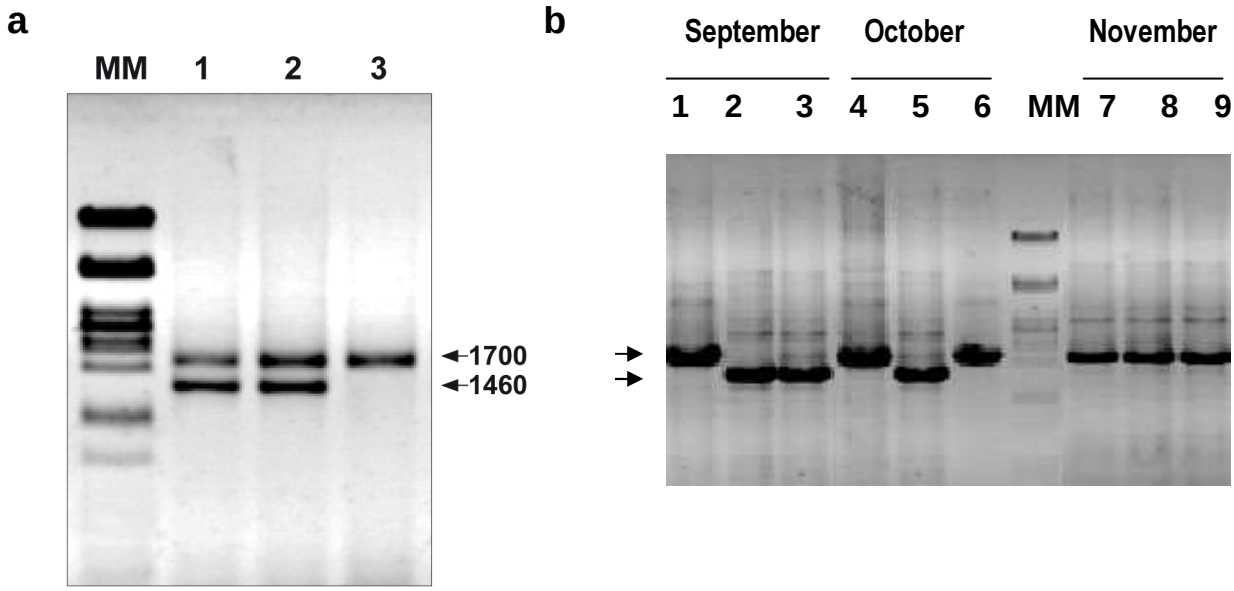

Figure 1. Agarose gel electrophoresis of DNA fragments amplified by PCR: **a)** from genomic DNA from representative surface samples collected at the Argentine Sea during spring (lane 1, in Septembrer; lane 2, in October; and lane 3, in November) using the primer pair EukA-EukB; **b)** from colonies of transformed *Escherichia coli* (colony-PCR) harbouring amplified DNA fragments mentioned in **a)**. Amplification produts from samples collected in September (lanes 1-3), in October (lanes 4-6) and in November (lanes 7-9). MM, 500-bp DNA ladder (MeBep Bioscience). Arrows indicate the two amplicons obtained. The 1,460-bp fragment corresponds to archaeal rDNA sequences. DNA fragments were visualized with ethidium bromide.

Figure 2. RFLP patterns obatined from 1,460-bp fragments. The PCR products were incubated with *Hae*III and the digestion products were separated by electrophoresis in agarose gels (2.5%). DNA fragments were visualized after ethidium bromide staining.

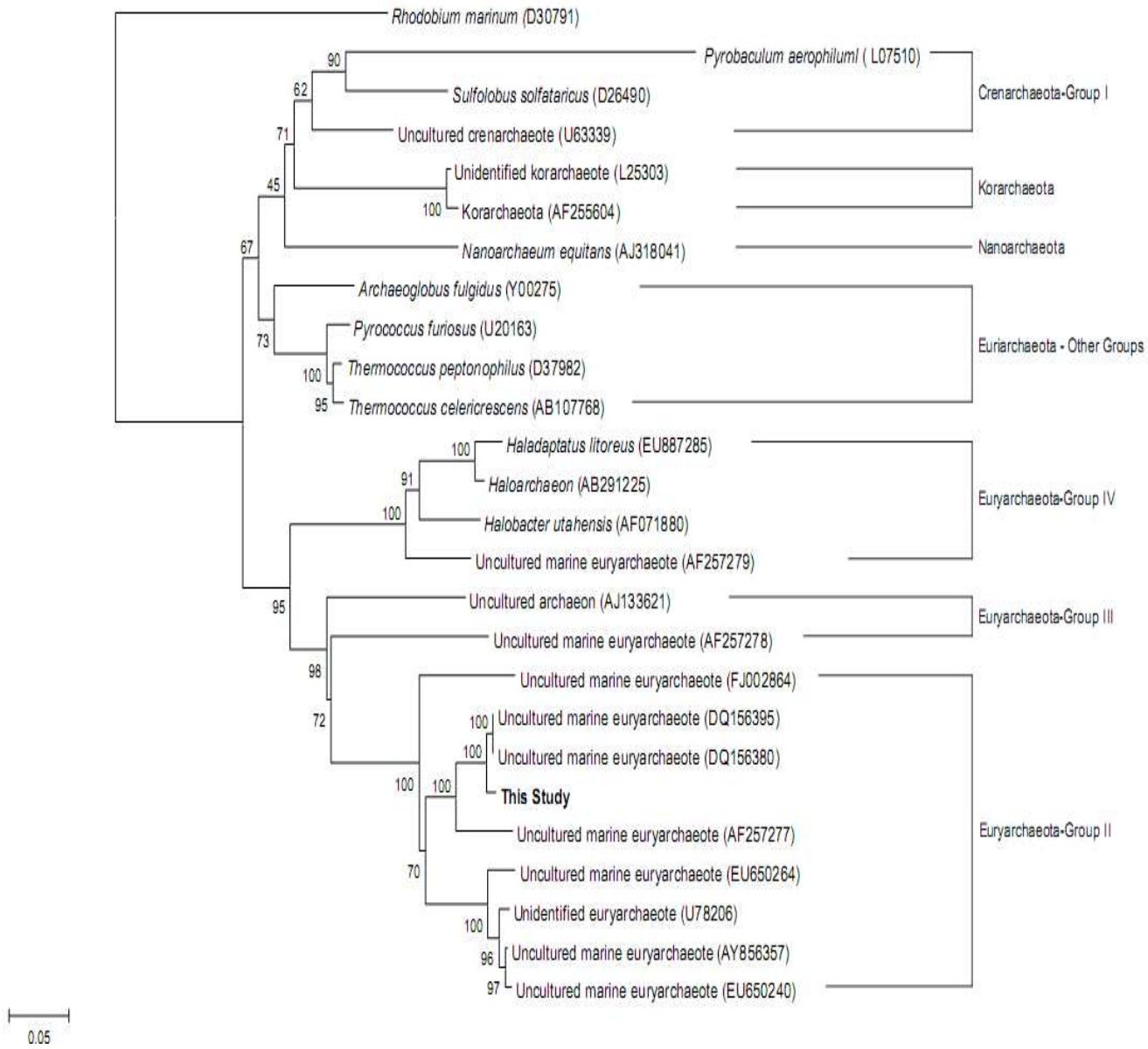

Figure 2. Phylogenetic 16S rDNA-based tree for partial sequences showing the phylogenetic position of novel archaeal 16S rRNA sequence identified from surface water samples of the Argentine Sea. All genomic information was downloaded from the Microbial Genomes resource of the National Center for Biotechnology Information (NCBI). Crenarchaeota-Group I: *Pyrobaculum aerophiluml* (L07510), *Sulfolobus solfataricus* (D26490), Uncultured crenarchaeote (U63339); Korarchaeota: Unidentified korarchaeote (L25303), Korarchaeota (AF255604); Nanoarchaeota: *Nanoarchaeum*

equitans (AJ318041); Euriarchaeota; Other Groups: *Archaeoglobus fulgidus* (Y00275), *Pyrococcus furiosus* (U20163), *Thermococcus peptonophilus* (D37982) *Thermococcus celericrescens* (AB107768); Euryarchaeota-Group IV: *Haladaptatus litoreus* (EU887285), Haloarchaeon (AB291225), *Halobacter utahensis* (AF071880), Uncultured marine euryarchaeote (AF257279); Euryarchaeota-Group III: Uncultured archaeon (AJ133621), Uncultured marine euryarchaeote (AF257278); Euryarchaeota-Group II: Uncultured marine euryarchaeote (FJ002864) Uncultured marine euryarchaeote (DQ156395); Uncultured marine euryarchaeote (DQ156380), Uncultured marine euryarchaeote (AF257277), Uncultured marine euryarchaeote (EU650264), Unidentified euryarchaeote (U78206), Uncultured marine euryarchaeote (AY856357), Uncultured marine euryarchaeote (EU650240). Marine alphaproteobacteria *Rhodobium marinum* (D30791) was used as an out group.

Table 1. Comparison of coverage of Archaea retrieved sequence-with commonly used non-degenerate Archaea primers included those for Eukarya (EukA and EukB) used at this study. Positions where each primer matches within the Archaea sequence HQ541865 are indicated.

Primer name	Sequence (5`-3`)	Commentary	Position in HQ541865 sequence of this study	Reference
Foward				
EukA	AACCTGGTTGATCCTGCCAGT		1	Medlin *et al*. (1988)
21F	TTCCGGTTGATCCYGCCGGA		None	DeLong (1992)
958R	YCCGGCGTTGAMTCCAATT		None	DeLong (1992)
1100A	TGGGTCTCGCTCGTTG		None	Embley *et al.* (1992)
Ab787F	ATTAGATACCCGGGTA		715	DasSarma and Fleischmann (1995)
PARCH 340f	CCCTACGGGG(C/***T***)GCA(***G***/C)CAG	***T*** and ***G*** to match	307	Ovreas *et al*. 1997
EK4F	CTGGTTGATCCTGCCAG		3	Robb *et al*. (1995).
A344F	ACGGGGTGCAGCAGGCGCGA		311	Casamayor *et al*. (2002)
958arcF	AATTGGANTCAACGCCGG	N=T	893	Huber *et al.* (2007)
Reverse				
EukB	TGATCCTTCTGCAGGTTCACCTAC		1436	Medlin *et al*. (1988)
ARCH 915R	GTGCTCCCCCGCCAATTCCT		849	Stahl and Amann (1991)
PARCH519r	TTACCGCGGC(***G***/T)GCTG	***G*** to match	448	Ovreas *et al*. 1997
PREA1100r	(T/C)GGGTCTCGCTCGTT(G/A)CC		None	In Ovreas *et al*. 1997
1048arcRmajor	CGRCGGCCATGCACCWC	R and W=A	976	Huber *et al.* (2007)